TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER P66141US0
		US APPLICATION NO (If known, see 37 CFR 1.5) 09/701583
INTERNATIONAL APPLICATION NO PCT/EP99/04013	INTERNATIONAL FILING DATE 10 June 1999	PRIORITY DATE CLAIMED 10 June 1998

TITLE OF INVENTION

A METHOD FOR STIMULATING THE IMMUNE SYSTEM

APPLICANT(S) FOR DO/EO/US

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Applicant herein submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ■ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ■ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ■ A proper Demand for Internatl. Preliminary Examination was made by the 19th month from earliest claimed priority date.
5. ■ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ■ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ■ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ■ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ■ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the Internatl. Preliminary Examination report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ■ Other items or information:

    International Search Report - EPO
    PCT/IB/301 Form
    PCT/IB/304 Form
    PCT/IB/308 Form
    International Preliminary Examination Report - with no annexes
    First Page of Publication

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	CALCULATIONS	PTO USE ONLY
17. ☒ The following fees are submitted:		
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Internatl. prelim. examination fee paid to USPTO (37 CFR 1.492 (a) (1)) . . $690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (2)) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) . . $710.00		
Neither international preliminary examination fee (37 CFR 1.492 (a) (3)) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO) **$1000.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (4)) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00		
Search Report prepared by the EPO or JPO (37 CFR 1.492 (a) (5)) **$860.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of $130.00 for furnishing the **oath or declaration** later than ☐ **20** ☒ **30** months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	**13** - 20 =	-0-	x $18.00	$	
Independent Claims	**2** - 3 =	-0-	x $80.00	$	
Multiple Dependent Claim(s) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 990.00	
Reduction by 1/2 , Applicant qualifies for Small Entity Status.				$	
SUBTOTAL =				$ 990.00	
Processing fee of $130 for furnishing the **English translation** later than ☐ **20** ☐ **30** months from the earliest claimed priority date (37 CFR 1.492(f))				$	
TOTAL NATIONAL FEE =				$ 990.00	
Fee of $40.00 for recording the enclosed **assignment** (37 CFR 1.21(h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31).				$	
TOTAL FEES ENCLOSED =				$ 990.00	
				Amt. to be refunded:	$
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a. ☒ A check in the amount of $ 990.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. **06-1358** in the amount of $ --- to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge my account any additional fees set forth in §1.492 during the pendency of this application, or credit any overpayment to Deposit Account No. **06-1358**. A duplicate copy of this sheet is enclosed.

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JPH&S 3/95

A Method for Stimulating the immune system

Two different approaches have been used in the prior art to enhance the immune response against neoplastic cells. One approach uses the addition of cytokines like interleukin-2 (IL-2) or transfection of tumor cells and/or immune cells with genes coding for cytokines like IL-2 or other proteins enhancing the immune response like transfection of tumor cells with lymphotactin or like transfection of T-lymphocytes with CD-40 Ligand.

The second approach uses the inhibition of immunosuppressive molecules to enhance the body's immune response to tumor cells. Thus, J. NEUROSURG. 78 (1993) 944-51, Jachimczak et al. (1993) and WO 94/25588, Schlingensiepen et al. (1994) teach the use of antisense oligonucleotides targeted to TGF-ß to reverse tumor-induced immunosuppression.

Several documents in the prior art teach that a combination of these two approaches is either not efficacious or is not beneficial over use of one of the two approaches used alone.

Thus, CANCER BIOTHER. 8(2), 1993, 159 - 170, Gridley et al., as well as CANCER BIOTHER. 9(4), 1994, 317-327, Mao et al., both teach that a combination of anti-transforming growth factor-beta antibody with IL-2 does not cause significant antitumor effects.

Furthermore, PROC. NATL. ACAD. SCI 93, (1996), 2909-2914, Fakhrai et al., teaches that a combination of transfection with genes encoding antisense sequences to transforming growth factor beta (TGF-ß) TGF-ß mRNA with transfection of IL-2 into tumor cells does not increase the immune response against the tumor compared to transfection with TGF-ß antisense alone.

Surprisingly, in contrast, certain combinations of stimulators and inhibitors are more efficacious than either approach alone.

The present invention discloses a medicament comprising a combination of

- at least one inhibitor of the effect of a substance negatively effecting an immune response, the substance selected from the group consisting of TGF-ß and its receptors, VEGF and its receptors, interleukin 10 (IL-10) and its receptors, PGE_2 and its receptors, wherein the inhibitor has a molecular weight of less than 100 kDa and

- at least one stimulator positively effecting an immune response.

In a preferred embodiment, the inhibitor is inhibiting the synthesis or function of molecules suppressing or downregulating or negatively affecting the immune response. The inhibitor can be an oligonucleotide which may function as an antisense nucleotide or a ribozyme or it may be an antibody fragment derived from an antibody e.g. a fab-fragment or a single chain antibody.

Preferably, the stimulator is positively effecting the immune response by increasing presentation of antigens and/or enhancing proliferation and/or function of immune cells.

In a preferred embodiment, the stimulator is enhancing the synthesis or function of molecules stimulating, enhancing, upregulating and/or positively regulating the immune response. In particular, the stimulator is stimulating and/or enhancing the synthesis and/or the function of factors such as GM-CSF, SCF, CSF, IFN-γ, FLT-3-ligand as well as monocyte chemotatic proteins (MCP-1), interleukin-2, interleukin-4, interleukin-12 and/or interleukin-18 or is one of the mentioned interleukins or is selected from the group consisting of viruses, viral antigens, antigens expressed in tumor cells or pathogens but not in normal cells, organspecific antigenes expressed in affected organs which are not essential for the organism, e. g. prostate, ovary, breast, melanine producing cells.

The stimulators are preferably selected from

a) Chemokines, including lymphotactin and/or immune cell attracting substances and/or

b) viruses and/or parts of viruses, including retroviruses, adenoviruses, papillomaviruses, Epstein-Barr-Vriuses, Viruses that are non-pathogenic including Newcastle-Disease virus, Cow-pox-virus and/or

c) autologous and/or heterologous MHC-Molecules and/or

d) molecules involved in antigen processing and/or

e) molecules involved in antigen presentation and/or

f) molecules involved in mediating immune cell effects and/or

g) molecules involved in mediating immune cell cytotoxic effects and/or

h) molecules involved in antigen transportation and/or

i) co-stimulatory molecules

j) peptides enhancing recognition by immune cells and/or cytotoxic effects of immune cells

k) the peptides containing one or more amino acids differing between a protein in the target cell from the other cells within an organism

l) the peptides according to j) being

- Peptides containing one or more mutations and/or amino acid substitutions of the ras protein amino and/or

- Peptides containing one or more mutations and/or amino acid substitutions of the p53 protein and/or

- Peptides containing one or more mutations and/or amino acid substitutions of the EGF-Receptor protein and/or

- Peptides containing one or more mutations and/or amino acid substitutions of fusion peptides and/or fusion proteins and/or

- Peptides containing one or more mutations and/or amino acid substitutions and/or amino acid substitutions caused by gene rearrangements and/or gene translocations and/or

- Peptides containing one or more mutations and/or amino acid substitutions of the retinoblastoma protein and/or

- Peptides containing one or more mutations and/or amino acid substitutions of proteins coded by oncogenes and/or protooncogenes and/or

- Peptides containing one or more mutations and/or amino acid substitutions of proteins coded by anti-oncogenes and/or tumor suppressor genes and/or

- Peptides derived from proteins differing in the target cell by one or more amino acids from the proteins expressed by other cells in the same organism and/or

- Peptides derived from viral antigens and/or coded by viral nucleic acids and/or

- Peptides derived from proteins expressed in a diseased organ but not in the nervous system, muscle, hematopoetic system or other organs essential for survival. Diseased organs are e. g. prostate, ovary, breast, melanine producing cells and the like.

m) tumor cell extracts and/or tumor cell lysates and/or adjuvants,

n) fusion cells of dendritic and tumor cells.

These fusion cells are hybridoma cells derived from a mixture of dentritic cells and tumor cells. Dentritic cells are generated e. g. by treatment of PBMC with GM-CSF and IL-4 or a mixture of GM-CSF, IL-4 and IFN-γ or FLT-3 ligand. Fusion of dendritic cells with tumor cells can be achieved e. g. using PEG (polyethylene glycole) or electrofusion.

Surprisingly, treatment of PBMC with VEGF-oligonucleotides enhanced the number and/or effectiveness of dendritic cells.

In one embodiment of the invention the inhibitor is an oligonucleotide. Preferably the oligonucleotides of Fig. 1 are useful in the medicament of the present invention.

In a further embodiment, the invention provides oligonucleotides having one of the sequences given in figure 1-2 to 1-4.

Also oligonucleotides having 1 to 10 additional nucleotides at the 5′- or 3′- end are part of the invention.

Oligonucleotide sequences used for transfection are usually much longer sequences than those used for antisense oligonucleotides, which usually do not exceed 30 bases in length and are applied as short single-stranded sequences and are not integrated into a vector system.

Since transfected sequences are usually much longer than oligonucleotides, if cross inhibition of different members of a protein family would occur with the antisense technology, such cross inhibition of other mRNAs than the target mRNA, is much more likely with transfected antisense sequences, compared to oligonucleotides. However, Cell Growth Differ, Vol. 6(12), February 1995, pages 1635 - 1642, Huang, F. et al.teaches "only the K6 transfectant exhibited 39 and 33% respectively of the levels or TGF beta1 mRNA and active secreted TGF beta1 protein of the parental line. K6 exhibited no change in TGF beta2 expression and TGF beta3 expression was not detected in either parental or transfectant cell line."

It was therefore surprising to find oligonucleotides according to this invention, which were able to significantly reduce expression of both, TGF-$ß_1$ as well as TGF-$ß_2$ e. g. TGF-ß1-14, TGF-ß1-15, TGF-ß-17-c-2260, TGF-ß-123-2262, TGF-ß-23-2268, TGF-ß2-4, TGF-ß2-14, TGF-ß2-15, TGF-ß2-9, TGF-ß2-14/1, TGF-ß2-14/2, TGF-ß1-136. Furthermore surprisingly oligonucleotides were designed, which were

able to significantly reduce expression of TGF-$ß_2$ as well as TGF-$ß_3$.

Surprisingly even oligonucleotides were found, which were able to significantly reduce expression of TGF-$ß_2$ as well as TGF-$ß_1$ and TGF-$ß_3$, e. g. b1-N17, b1-N14, b1-N24, TGF-ß2-9, TGF-ß2-14, TGF-ß-2-15, TGF-ß-17-c-2260, TGF-ß-12-9/20-2261, TGF-ß-123-2262, TGF-ß-12-9/22-2263, TGF-ß-23-2268, TGF-ß1-98-11, TGF-ß1-98-23, TGF-ß3-98-7, TGF-ß3-98-10, TGF-ß-1-rwk-5, TGF-ß-3-rwk-2, TGF-ß-1-rwk-5, TGF-ß-3-rwk-9, TGF-ß-3-rwk-23, TGF-ß1-3, TGF-ß1-10.

Thus oligonucleotides which are effective against expression of at least two of TGF-$ß_1$, TGF-$ß_2$ and/or TGF-$ß_3$ are also part of the invention.

These findings were also surprising in view of the fact that sequence comparison between the mRNAs of TGF-$ß_2$, TGF-$ß_1$ and TGF-$ß_3$ showed that not a single sequence of 20 bases in length could be found that would be identical within the three different mRNAs. Even if such a hypothetical sequence had really existed, inhibition of the three mRNAs by such a hypothetical consensus sequence would have been extremely unlikely, since it is well known in the art that only a small minority of antisense sequences complementary to a certain mRNA actually exert a so-called antisense effect, *i.e.* inhibit expression of the respective protein.

Endothelial synthesis of monocyte chemotactic protein-1 (MCP-1) has been implicated in the regulation of monocyte recruitment for extravascular pools both under physiological and inflammatory conditions.

MCP-1 antisense oligonuclotides were able to modulate monocyte infiltration and were thus anti-inflammatoric.

These antisense-oligonucloetides are useful for the treatment of inflammatory diseases e.g. asthma, morbus crohn, collitis ulcerosa, diabetes, glomerulonephritis, acute respiratory distress syndrome and artherosclerotic plaque formation.

In a preferred embodiment of the invention the oligonucleotides and/or ribozymes and/or nucleic acids have modifications at the bases, the sugars and/or the phosphate moieties of the oligonucleotides.

In a further preferred embodiment of the invention the oligonucleotides and/or ribozymes and/or nucleic acids have modifications wherein the modifications are phosphorothioate (S-ODN) internucleotide linkages and/or methylphosphonate internucleotide linkages and/or phosphoramidate linkages and/or peptide linkages and/or 2'-O-derivatives, such as 2′-O-methyl or 2′-O-methoxyethoxy modifications of the sugar and/or modifications of the bases.

In a further preferred embodiment of the invention the oligonucleotides and/or ribozymes and/or nucleic acids are coupled to or mixed with folic acid, hormones, steroid hormones such as oestrogene, progesterone, corticosteroids, mineral corticoids, peptides, proteoglycans, glycolipids, phospholipids, polyethylene imine or other poly cations and derivatives therefrom.

Furthermore, the present invention provides a method of treating hyperproliferative diseases, neoplasms or infectious diseases by administering a medicament of the invention to patients in need thereof. The method is especially useful for the treatment of leukemia, non-hodgkin lymphoma, hodgkin lymphoma, bronchial carcinoma, esophageal carcinoma, colorectal carcinoma, gastric carcinomas, intestinal tumors, hepatic tumors, gall bladder and gallduct carcinomas, pancreatic carcinoma, anal carcinoma, breast cancer, ovarian carcinoma, cervial carcinoma, endometrium carcinoma, prostatic carcinoma, bladder carcinoma, malignant melanoma, brain tumors, and sarcomas.

The necessary doses of the medicament of the present invention depend on the disease and the severity of the disease. Whereas higher levels are more effective, they often have a higher degree of side effects. Suitable doses are selected to obtain concentrations of the oligonucleotides in the range of 0.1 to 10 µmol/l and concentrations of the cytokines in the range of 10 to 1.000 U/ml in the patient blood.

In a preferred embodiment of the invention the inhibitor of the effect of a substance negatively effecting an immune response is applied locally to a tumor or other pathologically affected site or organ and the stimulator positively effecting an immune response is applied systemically (e.g. i.v. or s.c. or orally).

In another preferred embodiment of the invention the inhibitor of the effect of a substance negatively effecting an immune response is applied systemically (e.g. i.v or s.c. or orally) to the tumor and the stimulator positively effecting an immune response is applied locally to a tumor or other pathologically affected site or organ.
In another preferred embodiment of the invention the inhibitor of the effect of a substance negatively effecting an immune response is applied systemically (e.g. i.v. or s.c. or orally) to the tumor and the stimulator positively effecting an immune response is applied systemically (e.g. i.v. or s.c. or orally).

In another preferred embodiment of the invention the inhibitor of the effect of a substance negatively effecting an immune response is applied locally to a tumor or other pathologically affected site or organ and the stimulator positively effecting an immune response is applied locally to a tumor or other pathologically affected site or organ.

Fig. 1 shows oligonucleotides useful in the present invention.

Fig. 2A shows effects of oligonucleotides (f.c. 5 µM) on TGF-ß2 secretion in glioma cells in 10% MEM Dulbecco medium (3 day incubation with oligonucleotides).

Fig. 2B shows effects of oligonucleotides (f.c. 5 µM) on TGF-ß1 secretion in PBMC in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 3A shows effects of oligonucleotides (f.c. 5 µM) on TGF-ß1 secretion in PBMC in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 3B shows effects of oligonucleotides (f.c. 5 µM) on TGF-ß2 secretion in glioma cells in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 4A shows TGF-ß1 concentration (ELISA) in glioma cells (3 day incubation with oligonucleotides).

Fig. 4B shows TGF-ß2 concentration (ELISA) in glioma cells (3 day incubation with oligonucleotides).

Fig. 5 shows lysis of tumor-cells: LAK-Cytotoxicty, Ratio of glioma-cells/PBMC: 1 : 20.

Fig 6A shows dendritic cells generated from PBMC (% of control). Cytokines: GM-CSF (400 U/ml) + IL-4 (300 U/ml).

Fig. 6B shows lysis of tumor-cells: Effects of 5 µM VEGF-Antisense-Oligos on LAK-Cytotoxicty. Ration of tumor-cells/DC/PBMC was 1 : 5 :20.

Fig. 7A shows effects of oligonucleotides (f.c. 5 µM) on TGF-ß1 secretion in PBMC in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 7B shows effects of oligonucleotides (f.c. 5 µM) on TGF-ß2 secretion in tumro cells in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 8 shows lysis of tumor-cells: Effects of oligonucleotides on LAK-Cytotoxicty. Ration of tumor-cells/PBMC was 1 : 20.

Examples

Preparation of PBMC and tumor cells

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood of healthy donors by standard Ficoll-Hypaque gradient centrifugation. Briefly, heparinized blood was mixed with equal volumes of complete medium (CM: RMPI 1640 medium supplemented with 10% (v/v) fetal calf serum and 1 mM L-Glutamine) and layered onto a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient. After centrifugation at 400g for 30 min at room temperature, PBMCs banded at the plasma-Ficoll interface were recovered, washed tree times and resuspended in complete medium. Cell viability, as determined by Trypan blue exclusion, was greater than 97%.

Human glioma cell lines were established from tumor specimens of patients with anaplastic astrocytoma (WHO Grad III) or from glioblastoma (WHO Grad IV).

Measurement of cell proliferation

For PBMC-proliferation assays (3H-thymidine incorporation and cell counting), freshly isolated PBMCs were cultured for 72h in 96-well round-bottom plates (Nunc, Copenhagen, Denmark) at a final concentration (f.c.) of 10^5 cells/well (100 µl CM). For cell number determination the cells were counted by hemacytometer. Cell viability was determined by trypan blue staining. Treated and untreated cells showed 95-100% viability after 72h *in vitro* growth (with or without S-ODN).

For the tumor proliferation experiments 10^4/100 µL glioma cells were seeded into 96-well flat-bottom plates (Nunc, Denmark) and incubated with cytokines and/or oligonucleotides. The DNA synthesis rate was measured by a standard 3H-thymidine incorporation assay and determination of cell number was perfoimed as described above.

Quantification of TGF-ß1 protein in culture supernatants by enzyme-linked immunosorbent assay (ELISA)

The culture medium was harvested after 3 days, cleared of cellular components by centrifugation, filtered and stored at -70°C until processed further. TGF-ß1 and TGF-ß2 concentrations were measured after acidification of supernatants by TGF-ß1 and TGF-ß2 ELISA (R&D Systems, Minneapolis, USA) in duplicates, as recommended by the manufacturer.

Figures 1 - 4 and 7 show the effect of oligonucleotides on the TGF-ß secretion in cells. The concentration of the TGF-ß is reported as an optical density. The higher the optical density the higher is the concentration of the TGF-ß.

Figure 1A and 1B shows the effect of the oligonucleotides on the TGF-ß secretion. Control oligos (GAA GGA ATT ACC ACT TTC) have no effects whereas the oli-

gonucleotides shown in the figures reduce the secretion of TGF-ß. The oligos in figure 1 are more effective against TGF-ß1.

Figure 2 shows further oligos and their effects on TGF-ß secrection. TGF-ß-14 is especially effective against the secrection of TGF-ß1 and -ß2.

Figure 3 shows further oligonucleotides being effective against secretion of TGF-ß1 and -ß2. These oligonucleotides are more effective against TGF-ß2 but are also effective against TGF-ß1.

Figure 8 shows a supra additive effect on tumor cell cytotoxicity by a combination of 2 µM each of a TGF-ß1 and TGF-ß2 antisense oligonucleotide compared to a single 5 µM dose of either oligonucleotide.

CARE-LASS (calcein-release-assay) to measure cytotoxic PBMC activity

A standard calcein-release-assay (CARE-LASS assay) to determine cytotoxic activity of PBMC was employed as described by Lichtenfels, R., Biddison, W.E., Schulz, H., Vogt, A.B. and R. Martin. CARE-LASS (calcein-release assay), an improved fluorescence-based test system to measure cytotoxic lymphocyte activity. J. Immunol. Meth., 172: 227-239, 1994.

Target and Effector cells

At the day of the assay malignant glioma were harvested, washed twice in 5% FCS /PBS and incubated with Calcein-AM (Molecular Probes, USA) for 30min in 37°C. Labeled target cells were washed twice in 5% FCS/PBS, adjusted to 100 000 / ml, and plated into 96-well U-shaped microtiter plates (Nunc, Dennmark) at the final volumen of 100uL/well.

PBMC were washed with 5% FCS/PBS and adjusted to final concentration of 1-10 Mio cells /ml.

Cells were treated with cytokines and oligodeoxynucleotides as described in the individual experiments.

Assay

To measure CTL activity effector cells were plated into 96-well U-shape microtiter plates at Target : Effector Ratios of 1:10 - 1:100. To measure spontanous release and total release of calcein, wells were preloaded with 200uL 5%FCS/PBS or 200uL lysis buffer (50mM sodium-borate, 0.1% Triton, pH 9.0) respectively. After incubating the plate for 4 h at 37°C in an incubator, 100uL of supernatans were transferred into new wells and measured employing an automated fluorescence scanner (Titertek Fluoroskan II, Germany). Both for excitation and for emission, filter settings 2 were chosen (ex 2 – 485nm, em 2 –538 nm). The percent of cytotoxicity was determined from the following equation:

$$\frac{\text{F/CTL assay - F spontaneous release}}{\text{F total lysis - F spontanous release}} \times 100 = \%\ \text{cytotoxicity}$$

In one set of experiments, glioma cells, denritic cells (DC) and PBMC were co-cultured. In these experiments DC were generated from PBMC using the cytokines GM-CSF and IL4. Cells were further treated with antisense VEGF-oligonucleotides according to the invention or with no oligonucleotides as control experiments. Tumor cells were also treated with the cytokines GM-CSF and IL4 with or without oligonudeotides.

PBMC were only treated with oligonucleotides according to the invention, but not with the cytokines GM-CSF and IL4. oligos were used at a concentration of 5 μM unless indicated otherwise in the descriptions in the figures.

The CARE-LASS (calcein-release-assay) was used to measure cytotoxic PBMC activity.

In one set of experiments glioma cells and PBMC were treated either with a single oligonucleotide or with a combination of oligonucleotides. The single oligonucleotides were given at 5 μM concentration. In the combination experiment, each oligonucleotide was given at 2 μM concentration. Both, PBMC and tumor cells were incubated separately with the oligonucleotide(s) for 72 h.

The CARE-LASS (calcein-release-assay) was used to measure cytotoxic PBMC activity.

Claims

1. Medicament comprising a combination of

   - at least one inhibitor of the effect of a substance negatively effecting an immune response, the substance selected from the group consisting of TGF-ß and its receptors, VEGF and its receptors, interleukin 10 (IL-10) and its receptors, PGE_2 and its receptors, wherein the inhibitor has a molecular weight of less than 100 kDa and

   - at least one stimulator positively effecting an immune response.

2. The medicament of claim 1 wherein the inhibitor is inhibiting the synthesis or function of molecules suppressing or downregulating or negatively affecting the immune response.

3. The medicament of claim 1 wherein the inhibitor is an oligonucleotide.

4. The medicament according to claim 3 wherein the oligonucleotide is an antisense nucleotide and/or a ribozyme.

5. The medicament according to claim 3 wherein the oligonucleotides has a sequence according to figure 1.

6. The medicament according to claim 1, wherein the inhibitor is a fab-fragment or single chain antibody (scFv).

7. The medicament according to claim 1, wherein the stimulator is enhancing the synthesis or function of molecules stimulating, enhancing, upregulating and/or positively regulating the immune response.

8. The medicament according to claim 7, wherein the stimulator is stimulating and/or enhancing the synthesis and/or the function of factors such as GM-CSF, SCF, CSF, IFN, FLT-3-ligand, monocyte chemotatic proteins (MCP-1), interleukin-2, interleukin-4, interleukin-12 and/or interleukin-18 or is one of the mentioned interleukins or is selected from the groups consisting of viruses, viral antigens, antigens expressed in tumor cells or pathogens, but not in normal cells, organ specific antigens expressed in affected organs which are not essential for the organism or fusion cell of dendritic and tumor cells.

9. The medicament according to claim 1, wherein the medicament comprises two or more of the inhibitors and/or the stimulators.

10. An oligonucleotide having one of the sequences of figures 1-2 to 1-4 (No. 55 - 213).

11. The oligonucleotide according to claim 10 wherein each oligonucleotide is effective against expression of at least two of TGF-$ß_1$, TGF-$ß_2$ and/or TGF-$ß_3$ having the sequence.

12. A method of treating neoplasm or infectious diseases by administering a medicament according to claim 1 to a patient in need thereof.

13. A method according to claim 12 for the treatment of hyperproliferative diseases, leukemia, non-hodgkin lymphoma, hodgkin lymphoma, bronchial carcinoma, esophageal carcinoma, colorectal carcinoma, gastric carcinomas, intestinal tumors, hepatic tumors, gall bladder and gallduct carcinomas, pancreatic carcinoma, anal carcinoma, mastocarcinoma, ovarian carcinoma, cervial carcinoma, endometrium carcinoma, prostatic carcinoma, bladder carcinoma, malignant melanoma, brain tumors, and/or sarcomas.

1.	TGF-ß2-1	C ACA CAG TAG TGC A
2.	TGF-ß2-2	GC ACA CAG TAG TGC
3.	TGF-ß2-3	GC TTG CTC AGG ATC TGC
4.	TGF-ß2-4	TAC TCT TCG TCG CT
5.	TGF-ß2-5	C TTG GCG TAG TAC T
6.	TGF-ß2-6	G TAA ACC TCC TTG G
7.	TGF-ß2-7	GT CTA TTT TGT AAA CCT CC
8.	TGF-ß2-8	GC ATG TCT ATT TTG TAA ACC
9.	TGF-ß2-9	CGG CAT GTC TAT TTT GTA
10.	TGF-ß2-10	G GCA TCA AGG TAC C
11.	TGF-ß2-11	CTG TAG AAA GTG GG
12.	TGF-ß2-12	AC AAT TCT GAA GTA GGG T
13.	TGF-ß2-13	T CAC CAA ATT GGA AGC AT
14.	TGF-ß2-14	GCT TTC ACC AAA TTG GAA GC
15.	TGF-ß2-15	CTG GCT TTT GGG TT
16.	TGF-ß2-16	T CTG ATA TAG CTC AAT CC
17.	TGF-ß2-17	T CCT AGT GGA CTT TAT AG
18.	TGF-ß2-18	T TTT TCC TAG TGG ACT
19.	TGF-ß2-19	C AAT TAT CCT GCA CAT TTC
20.	TGF-ß2-20	GC AAT TAT CCT GCA CA
21.	TGF-ß2-21	GC AGC AAT TAT CCT GC
22.	TGF-ß2-22	TG GCA TTG TAC CCT
23.	TGF-ß2-23	TG TGC TGA GTG TCT
24.	TGF-ß2-24	CC TGC TGT GCT GAG TG
25.	TGF-ß2-25	C TTG GGT GTT TTG C
26.	TGF-ß2-26	T TTA GCT GCA TTT GCA AG
27.	TGF-ß2-27	G CCA CTT TTC CAA G
28.	TGF-ß2-14/1	CTT TCA CCA AAT TGG AAG
29.	TGF-ß2-14/2	CAC CAA ATT GGA AGC
30.	TGF-ß2-14/3	TCA CCA AAT TGG AAG C
31.	TGF-ß2-15/1	CTC TGG CTT TTG GG
32.	TGF-ß2-9/1	CGG CAT GTC TAT TTT G
33.	TGF-ß1-1	CGA TAG TCT TGC AG
34.	TGF-ß1-2	GTC GAT AGT CTT GC
35.	TGF-ß1-3	CTT GGA CAG GAT CT
36.	TGF-ß1-4	CCA GGA ATT GTT GC
37.	TGF-ß1-5	CCT CAA TTT CCC CT
38.	TGF-ß1-6	GAT GTC CAC TTG CA
39.	TGF-ß1-7	CTC CAA ATG TAG GG
40.	TGF-ß1-8	ACC TTG CTG TAC TG
41.	TGF-ß1-9	GTA GTA CAC GAT GG
42.	TGF-ß1-10	CAC GTA GTA CAC GA
43.	TGF-ß1-11	CAT GTT GGA CAG CT
44.	TGF-ß1-12	GCA CGA TCA TGT TG
45.	TGF-ß1-13	TGT ACT CTG CTT GAA C
46.	TGF-ß1-14	CTG ATG TGT TGA AGA ACA
47.	TGF-ß1-15	CTC TGA TGT GTT GAA G
48.	TGF-ß1-16	GGA AGT CAA TGT ACA G
49.	TGF-ß1-17	CAT GTC GAT AGT CTT GCA
50.	TGF-ß1-18	AGC TGA AGC AAT AGT TGG
51.	TGF-ß1-19	GTC ATA GAT TTC GTT GTG
52.	TGF-ß1-20	CTC CAC TTT TAA CTT GAG
53.	TGF-ß1-21	TGC TGT ATT TCT GGT ACA
54.	TGF-ß1-137	CGA TAG TCT TGC AG

Fig. 1-1

55.	b1-N17	TCC TCT TCG ACT GCT CTC
56.	b2-N14	CGA AGG TTA AAC CAC TTT CG
57.	b2-N24	GTG AGT CGT GTC GTC C
58.	TGF-ß2-98-1	CATCGTTGTCGTCG
59.	TGF-ß2-98-2	CGCTTCTTCCGCCG
60.	TGF-ß2-98-3	CGAAGGAGAGCCATTCG
61.	TGF-ß2-98-4	CGATGTAGCG
62.	TGF-ß2-98-5	CGTCAAATCG
63.	TGF-ß2-98-6	CGTAGTACTCTTCGTCG
64.	TGF-ß2-98-7	CGCGCTCGCAGGCG
65.	TGF-ß2-98-8	CGGCCGCCCTCCGGCTCG
66.	TGF-ß2-98-9	CGCGGATCGCCTCG
67.	TGF-ß2-98-10	GAGCGCGACCGTGAC
68.	TGF-ß-17-c-2260	ACC TCC TTG GCG TAG TA
69.	TGF-ß-12-9/20-2261	AGG GCG GCA TGT CTA TTT TG
70.	TGF-ß-123-2262	CAG AAG TTG GCA TTG TAC
71.	TGF-ß-12-9/22-2263	AGG GCG GCA TGT CTA TTT TGT A
72.	TGF-ß-23-2268	TGG GAC ACG CAG CAA GG
73.	TGF-ß1-98-1	CGGGGGCGGGGCGGGG
74.	TGF-ß1-98-2	CGGGGCGGGGCGGGGCG
75.	TGF-ß1-98-3	CGGCGCCGCCGAGGCGCCCG
76.	TGF-ß1-98-4	CCGAGGTCCTTGCGG
77.	TGF-ß1-98-5	CGGCGGTGCCGGGA
78.	TGF-ß1-98-6	CTCGGCGGCCGGTAG
79.	TGF-ß1-98-7	CGCTAAGGCG
80.	TGF-ß1-98-8	CCGCACAACTCCGG
81.	TGF-ß1-98-9	GCGAGTCGCTGG
82.	TGF-ß1-98-10	CGGTTGCTGAGGTATCG
83.	TGF-ß1-98-11	CCGGGAGAGCAACACGG
84.	TGF-ß1-98-12	CGCTTCTCG
85.	TGF-ß1-98-13	CCATTAGCACGCGGG
86.	TGF-ß1-98-14	CGGGCTCCG
87.	TGF-ß1-98-15	CCGGCCACCCGGTCGCGG
88.	TGF-ß1-98-16	CGAGCACGGCCTCG
89.	TGF-ß1-98-17	CGGGCAGCGGGCCGGGCG
90.	TGF-ß1-98-18	CGCGGATGGCCTCG
91.	TGF-ß1-98-19	CGATGCGCTTCCG
92.	TGF-ß1-98-20	CCCGCGGCCGGCGGG
93.	TGF-ß1-98-21	CGCAGCCCGGAGGGCG
94.	TGF-ß1-98-22	CGGCGCCCCCCG
95.	TGF-ß1-98-23	CGGCACTGCCGAGAGCGCG
96.	TGF-ß1-98-24	CGGGGATGAAGGCGGCG
97.	TGF-ß1-98-25	CGGGTCGGCGACTCCCG
98.	TGF-ß1-98-26	CGCCTGAGGGACGCCG
99.	TGF-ß1-98-27	AAGCGTCCCCGGCG
100.	TGF-ß1-98-28	CGCGGGGCAGCGTCGCG
101.	TGF-ß1-98-29	CCCCGCGCCTCCGG
102.	TGF-ß1-98-30	CGGCGGCGGCTCG
103.	TGF-ß1-98-31	CGCTCCGGGCCGAGGCCG
104.	TGF-ß1-98-32	CGGCCCCGCGGGCG
105.	TGF-ß1-98-33	CGGACGGGGCGTCC
106.	TGF-ß1-98-34	CGGCCGGGGCCCTCG

Fig. 1-2

107.	TGF-ß3-98-1	TCGAGCTTCCCCGA
108.	TGF-ß3-98-2	CCCGGAGCCGAAGG
109.	TGF-ß3-98-3	CCCGAGGAGCGGG
110.	TGF-ß3-98-4	ACGCAGCAAGGCGA
111.	TGF-ß3-98-5	CGGGTTGTCGAGCCG
112.	TGF-ß3-98-6	CGGCAGTGCCCCG
113.	TGF-ß3-98-7	CGGAATTCTGCTCG
114.	TGF-ß3-98-8	TTCGTTGTGCTCCG
115.	TGF-ß3-98-9	ATTCCGACTCGGTG
116.	TGF-ß3-98-10	ACGTGGGTCATCACCGT
117.	TGF-ß3-98-11	CGAAGAAGCG
118.	TGF-ß3-312	CCT AAT GGC TTC CA
119.	VEGF-98-1	CGGCCGCGGTGTGT
120.	VEGF-98-2	CGGGAATGCTTCCGCCG
121.	VEGF-98-3	CGGCTCACCGCCTCGGC
122.	VEGF-98-4	CACGTCTGCGGATC
123.	VEGF-98-5	CCCCGCATCGCATCAGGG
124.	VEGF-98-6	CGCCTTGCAACGCG
125.	VEGF-98-7	CCGACCGGGGCCGG
126.	VEGF-49	GTTCATGGTTTCGG
127.	VEGF-55	GCAGAAAGTTCATGG
128.	VEGF-188	GCTGATAGACATCC
129.	VEGF-190	GCGCTGATAGACAT
130.	VEGF-194	GTAGCTGCGCTGATAG
131.	VEGF-253	CTCGATCTCATCAG
132.	VEGF-255	ATGTACTCGATCTCATC
133.	VEGF-260	GAAGATGTACTCGATC
134.	VEGF-263	CTTGAAGATGTACTCG
135.	VEGF-292	GCATCGCATCAGGG
136.	VEGF-294	CCGCATCGCATCAG
137.	VEGF-422	CATTTGTTGTGCTGTAGG
138.	VEGF-434	GGTCTGCATTCACATTTG
139.	VEGF-441	CTTTGGTCTGCATTC
140.	VEGF-445	CTTTCTTTGGTCTGC
141.	VEGF-450	GCTCTATCTTTCTTTGG
142.	VEGF-455	GTCTTGCTCTATCTTTC
143.	VEGF-459	CTTGTCTTGCTCTATC
144.	VEGF-596	CATCTGCAAGTACGTTCG
145.	VEGF-598	CACATCTGCAAGTACGTT
146.	VEGF-600	GTCACATCTGCAAGTACG
147.	VEGF-600-2	CATCTGCAAGTACG
148.	VEGF-601	CACATCTGCAAGTAC
149.	VEGF-604	GTCACATCTGCAAG
150.	VEGF-607	CTTGTCACATCTGC
151.	VEGF-607-2	GGCTTGTCACATCTGC
152.	VEGF-610	CTCGGCTTGTCACATC
153.	VEGF-638	CTCCTTCCTCCTGC
154.	VEGF-766	GCT TGA AGA TGT ACCT CG
155.	VEGF-r-1062	CGT TGC TCT CCG ACG
156.	flt-1165	GAC ACG GCC TTT TCG
157.	flt-rm-2115	CCA GCA GCT GAC CAT GG
158.	flk1/kdr-m-2315	GAA ATC GAC CCT CGG
159.	MCP-1-Rec-A/B-571	GCA TGT TGT GGA TG
160.	MCP-1-1954	GCA GAG ACT TTC ATG C
161.	MCP-1-1955	ATA ACA GCA GGT GAC TGG

Fig. 1-3

162.	MCP-1-1956	GAA CCC ACT TCT GC
163.	MCP-1-2761	GAC ACT TGC TGC TG
164.	MCP-1-2762	CCA CTT CTG CTT GGG
165.	VEGF-703	CTG CAA GTA CGT TCG
166.	flt-1567	TCC CTT ATG ATG CCA GCA AGT G
167.	TGF-ß-Rec-I-796	CCA GCA ATG ACA GC
168.	TGF-ß-1-rwk-1	G GGA AAG CTG AGG C
169.	TGF-ß-1-rwk-2	T CGA GGG AAA GCT GA
170.	TGF-ß-1-rwk-3	C CTC GAG GGA AAG C
171.	TGF-ß-1-rwk-4	GG GCT GGT GTG GTG
172.	TGF-ß-1-rwk-5	GA ACA GGG CTG GTG TG
173.	TGF-ß-1-rwk-6	G AAC AGG GCT GGT G
174.	TGF-ß-1-rwk-7	AG AGC GCG AAC AGG
175.	TGF-ß-1-rwk-8	GA GAG CGC GAA CAG G
176.	TGF-ß-1-rwk-9	CGA GAG CGC GAA CAG
177.	TGF-ß-1-rwk-10	CCC CTG GCT CGG GGG
178.	TGF-ß-1-rwk-11	C CCT GGC TCG GGG
179.	TGF-ß-1-rwk-12	C CCC TGG CTC GGG G
180.	TGF-ß-1-rwk-13	TCC CCC TGG CTC GG
181.	TGF-ß-1-rwk-14	C TCC CCC TGG CTC G
182.	TGF-ß-1-rwk-15	TGC GCT TCC GCT TCA C
183.	TGF-ß-1-rwk-16	CC TCG ATG CGC TTC
184.	TGF-ß-1-rwk-17	G ATG GCC TCG ATG C
185.	TGF-ß-1-rwk-18	G GAT GGC CTC GAT GC
186.	TGF-ß-1-rwk-19	ATG GCC TCG ATG CGC TT
187.	TGF-ß-3-rwk-1	TC AGC AGG GCC AGG
188.	TGF-ß-3-rwk-2	GCA AAG TTC AGC AGG GC
189.	TGF-ß-3-rwk-3	GG CAA AGT TCA GCA GG
190.	TGF-ß-3-rwk-4	GT GGC AAA GTT CAG CAG G
191.	TGF-ß-3-rwk-5	GTG GCA AAG TTC AG
192.	TGF-ß-3-rwk-6	GAC CGT GGC AAA GTT CAG
193.	TGF-ß-3-rwk-7	AGA GAG GCT GAC CGT
194.	TGF-ß-3-rwk-8	GAC AGA GAG AGG CTG AC
195.	TGF-ß-3-rwk-9	A CAG AGA GAG GCT GA
196.	TGF-ß-3-rwk-10	GT GGA CAG AGA GAG G
197.	TGF-ß-3-rwk-11	CA AGT GGA CAG AGA GAG G
198.	TGF-ß-3-rwk-12	TCT TCT TGA TGT GGC C
199.	TGF-ß-3-rwk-13	CC CTC TTC TTC TTG ATG
200.	TGF-ß-3-rwk-14	C ACC CTC TTC TTC T
201.	TGF-ß-3-rwk-15	A TGG ATT TCT TTG GCA T
202.	TGF-ß-3-rwk-16	GGA TTT CTT TGG C
203.	TGF-ß-3-rwk-17	AA GTT GGA CTC TCT TCT C
204.	TGF-ß-3-rwk-18	TAA GTT GGA CTC TCT TCT
205.	TGF-ß-3-rwk-19	GAC CTA AGT TGG ACT C
206.	TGF-ß-3-rwk-20	T TTC TAG ACC TAA GTT GG
207.	TGF-ß-3-rwk-21	CT GAT TTC TAG ACC TAA G
208.	TGF-ß-3-rwk-22	G AAG CAG TAA TTG GTG T
209.	TGF-ß-3-rwk-23	GG AAT CAT CAT GAG G
210.	TGF-ß-3-rwk-24	GGG AAT CAT CAT GAG
211.	TGF-ß-3-rwk-25	G GTT GTC GAG CCG GT
212.	TGF-ß-3-rwk-26	GTC CTC CCA ACA TAG TA
213.	TGF-ß-3-rwk-27	GG GTC CTC CCA ACA

Fig. 1-4

A.
TGF-B1-14
TGF-B1-15
TGF-B17-c-2260
TGF-B-123-2262
TGF-B-23-2268
Control Oligo B
No Oligo
0
0,2
0,4
0,6
0,8
1
1,2
1,4
1,6
1,8
2
OD 450-570 nm
B.
TGF-B1-14
TGF-B1-15
TGF-B17-c-2260
TGF-B-123-2262
TGF-B-23-2268
Control Oligo B
No Oligo
0
0,05
0,1
0,15
0,2
0,25
0,3
OD 450-570 nm

Figure 2

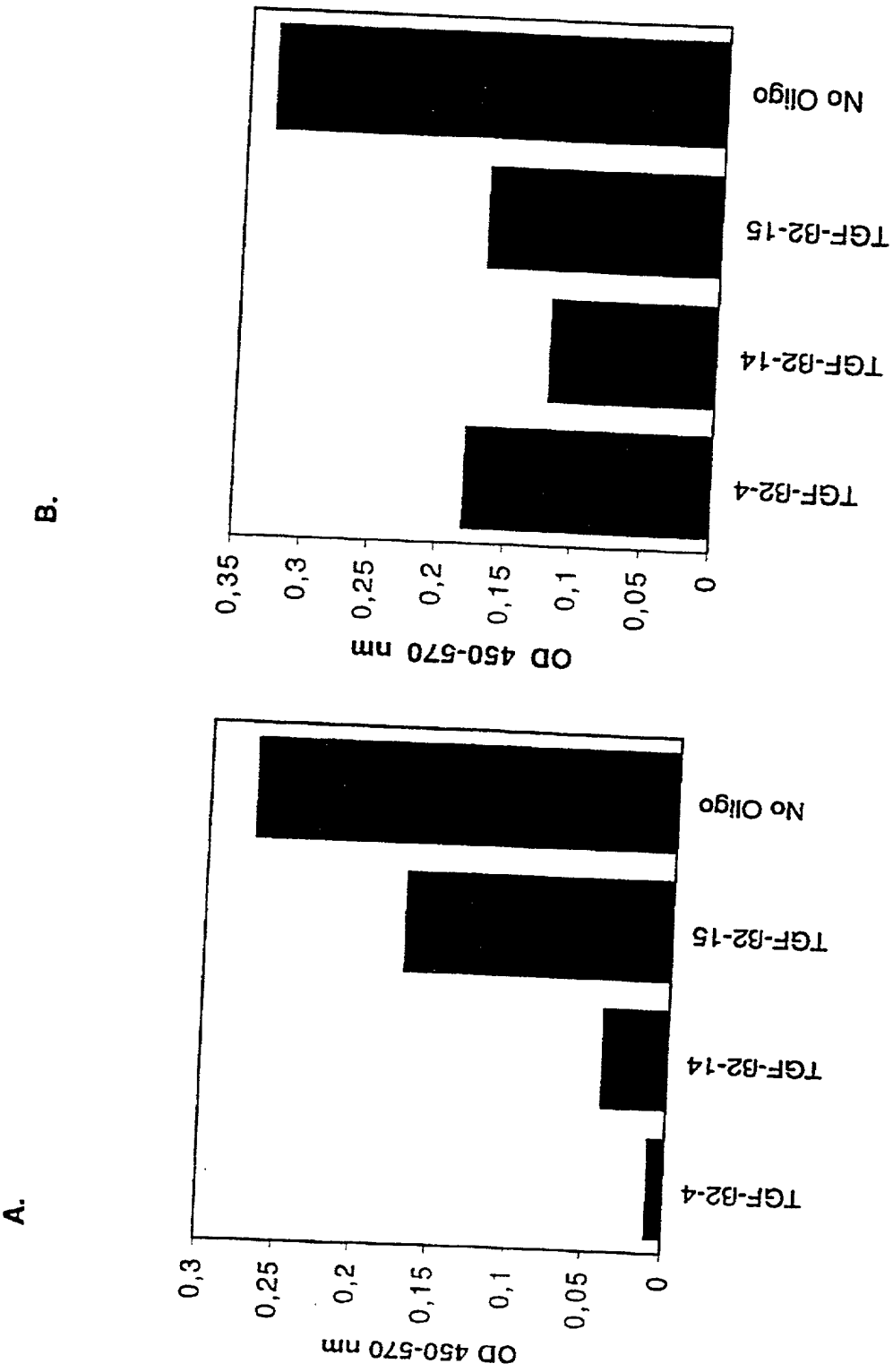
A.
TGF-β2-4
TGF-β2-14
TGF-β2-15
No Oligo
0
0,05
0,1
0,15
0,2
0,25
0,3
OD 450-570 nm
B.
TGF-β2-4
TGF-β2-14
TGF-β2-15
No Oligo
0
0,05
0,1
0,15
0,2
0,25
0,3
0,35
OD 450-570 nm

Figure 3

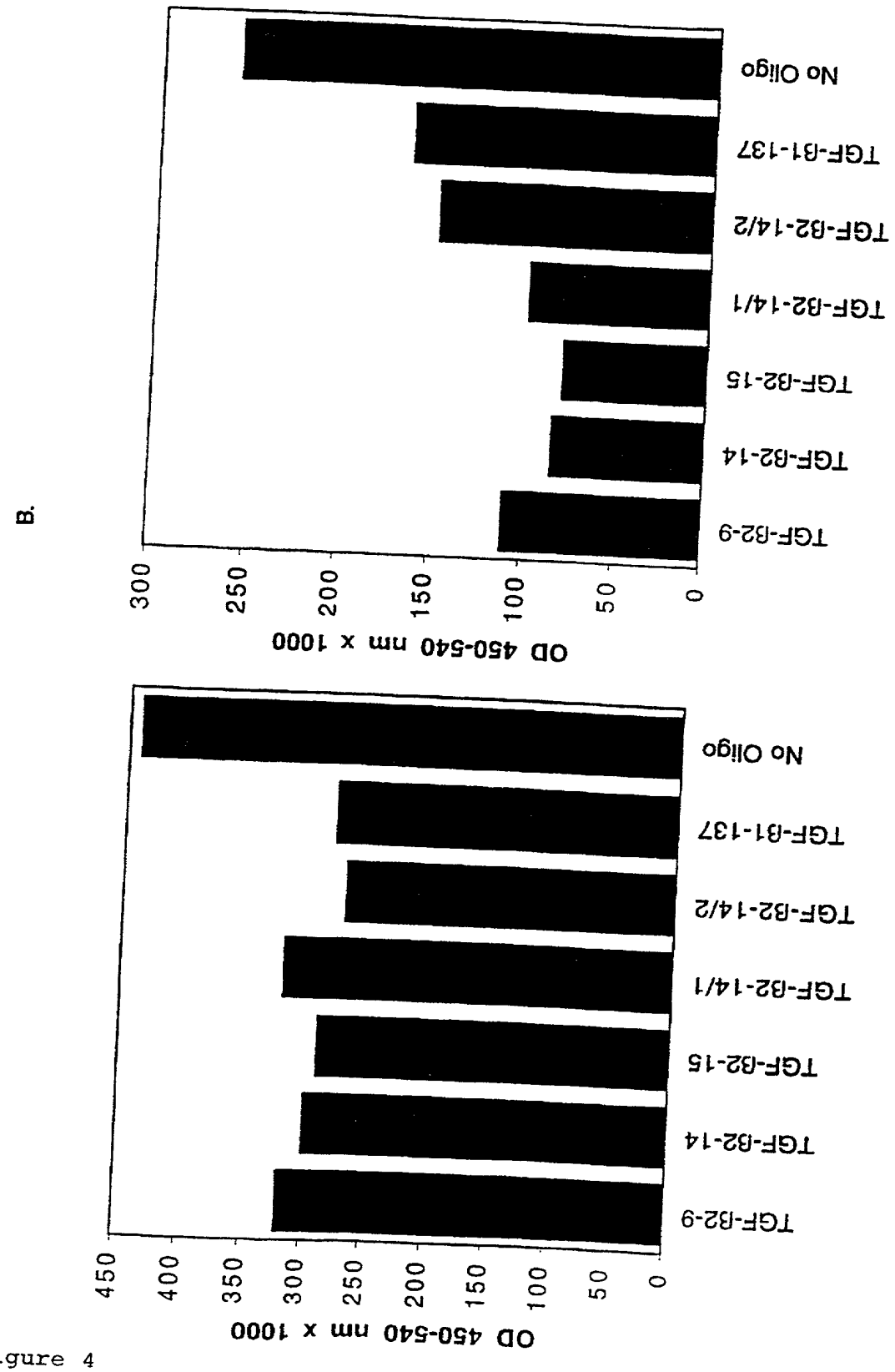
B.
TGF-β2-9
TGF-β2-14
TGF-β2-15
TGF-β2-14/1
TGF-β2-14/2
TGF-β1-137
No Oligo
0
50
100
150
200
250
300
OD 450-540 nm x 1000
A.
TGF-β2-9
TGF-β2-14
TGF-β2-15
TGF-β2-14/1
TGF-β2-14/2
TGF-β1-137
No Oligo
0
50
100
150
200
250
300
350
400
450
OD 450-540 nm x 1000

Figure 4

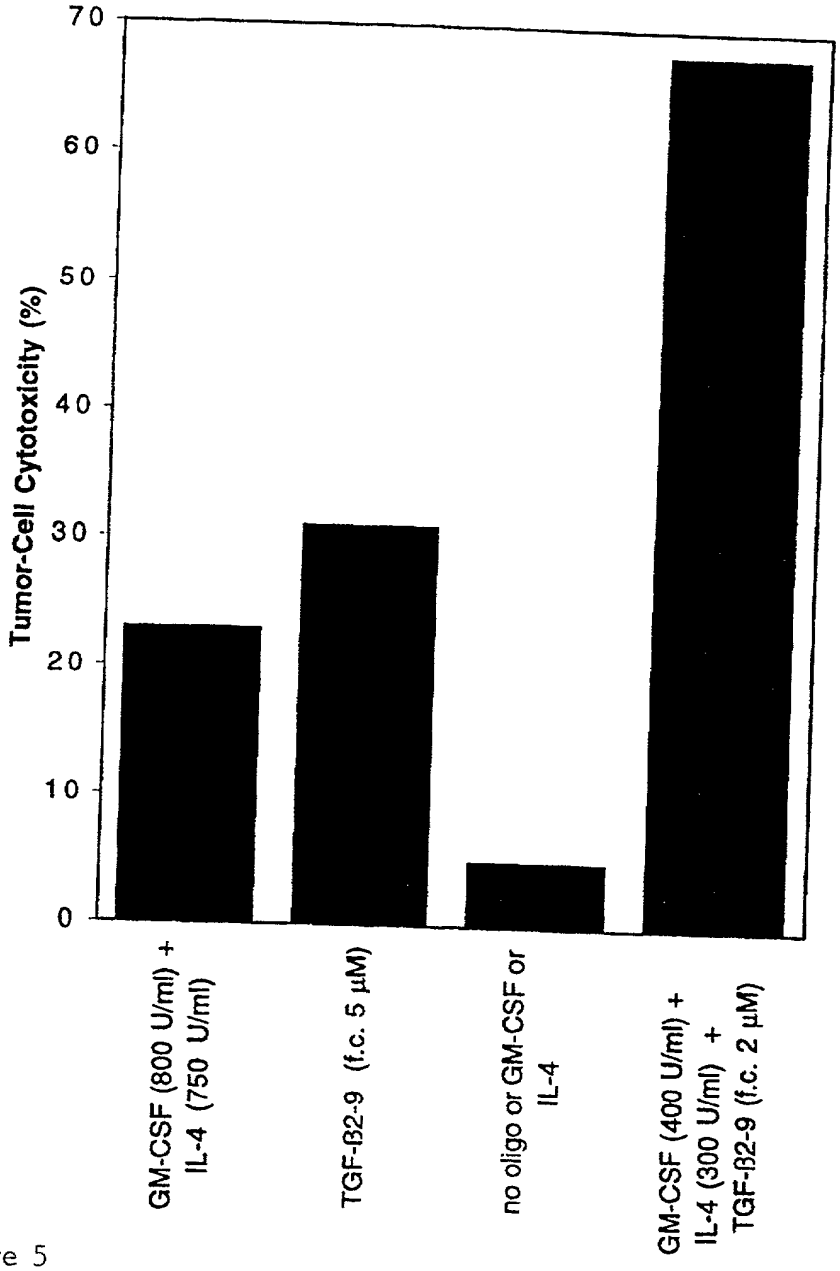
Tumor-Cell Cytotoxicity (%)
70
60
50
40
30
20
10
0
GM-CSF (800 U/ml) + IL-4 (750 U/ml)
TGF-β2-9 (f.c. 5 µM)
no oligo or GM-CSF or IL-4
GM-CSF (400 U/ml) + IL-4 (300 U/ml) + TGF-β2-9 (f.c. 2 µM)

Figure 5

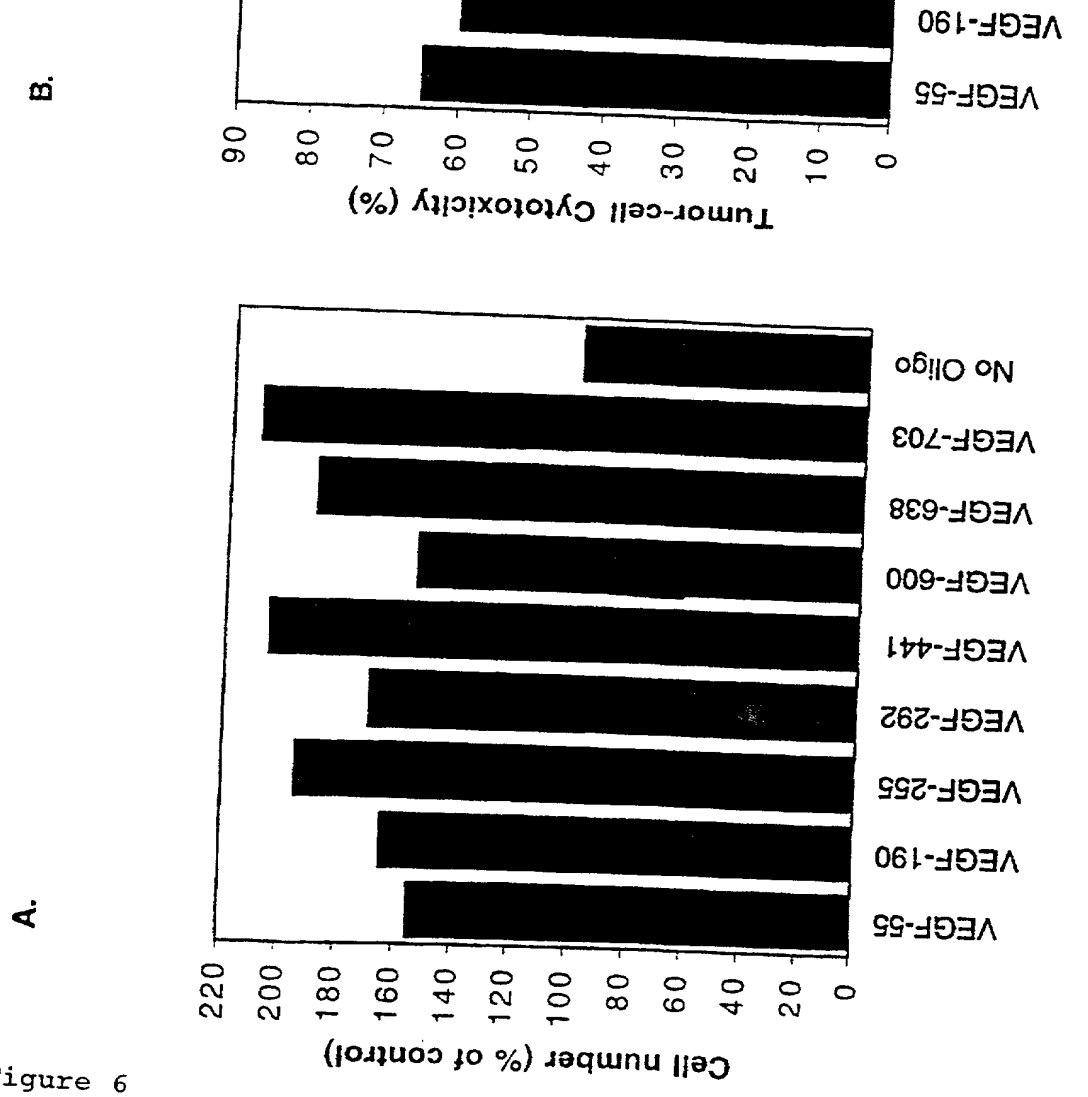

Figure 6

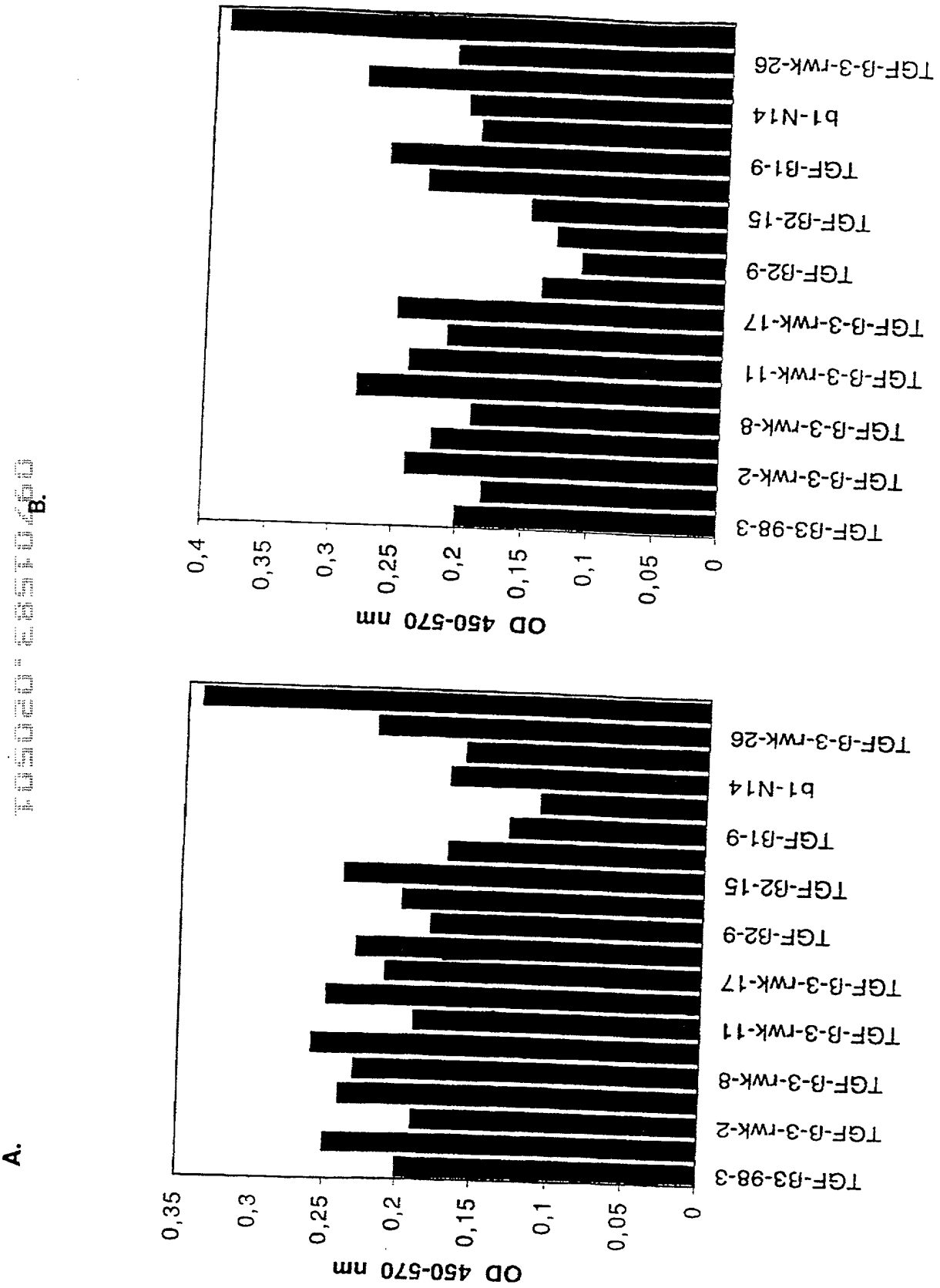
A.
TGF-B3-98-3
TGF-B-3-rwk-2
TGF-B-3-rwk-8
TGF-B-3-rwk-11
TGF-B-3-rwk-17
TGF-B2-9
TGF-B2-15
TGF-B1-9
b1-N14
TGF-B-3-rwk-26
0,35 0,3 0,25 0,2 0,15 0,1 0,05 0
OD 450-570 nm
B.
TGF-B3-98-3
TGF-B-3-rwk-2
TGF-B-3-rwk-8
TGF-B-3-rwk-11
TGF-B-3-rwk-17
TGF-B2-9
TGF-B2-15
TGF-B1-9
b1-N14
TGF-B-3-rwk-26
0,4 0,35 0,3 0,25 0,2 0,15 0,1 0,05 0
OD 450-570 nm

Figure 7

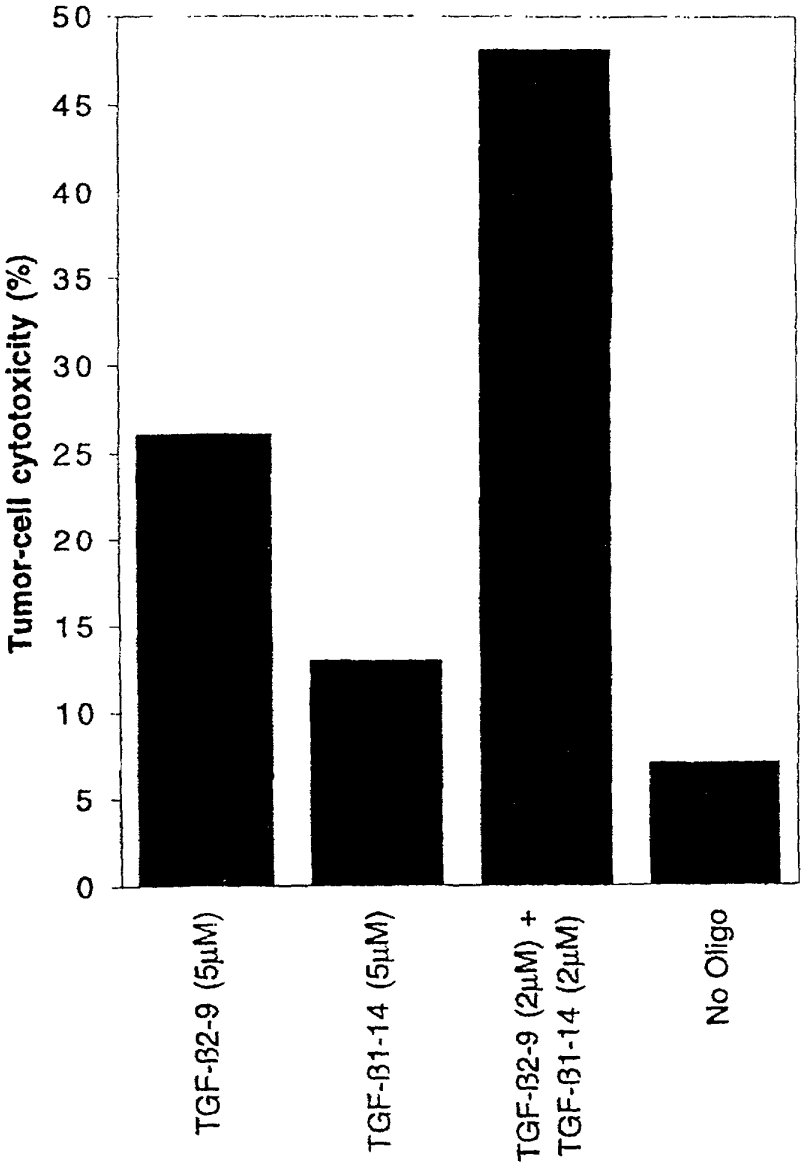
Tumor-cell cytotoxicity (%)
50
45
40
35
30
25
20
15
10
5
0
TGF-β2-9 (5μM)
TGF-β1-14 (5μM)
TGF-β2-9 (2μM) + TGF-β1-14 (2μM)
No Oligo

Figure 8